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Assessment of Cyfluthrin commercial formulation on growth, photosynthesis and catalase activity of green algae

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ABSTRACT

Aquatic environments of the pampasic region of Argentina are severely affected by agricultural contamination due to an increase in a glyphosate tolerant transgenic variety of soybean crops. The present study is aimed to determine the effects of a commonly used Cyfluthrin commercial formulation (CCF) on growth, some physiological and biochemical parameter of four species of green algae. Significant inhibition of algal growth was observed from 0.1 mg Cyf/l. 96 h IC_{50} were between 0.92 and 4.85 mg Cyf/l. CCF caused algicidal effects. Photosynthesis was stimulated by 50% in *Scenedesmus quadricauda* cultures exposed to the lowest concentration (hormesis). Algal photosynthesis inhibition was observed at higher concentrations with IC_{50} values between 1.7 and 8.9 mg Cyf/l. Similar toxicity endpoints were found as a consequence of applying the traditional methodology of short-term chronic toxicity test of 96 h of exposition and the methodology developed using the Clark type photosynthetic oxygen evolution method. CAT activity was significantly increased between 23% and 33% considering the four species, at a lower concentration than those affecting algal growth and photosynthesis, indicating a potential biomarker. Taking into account that the extent of the soybean crops in the region is about fourteen million hectares, the improvement and extension of environmental tools for early detection of the action of pesticides on this essential group of organisms are discussed.

1. Introduction

The pyrethroids insecticides are among the most powerful, being synthetic derivatives of natural products from flowers of *Chrysanthemum cinerariaefolium*. Compared with previous pyrethroids, recent products have very low solubility (10–80 μg/l) and greater hydrophobicity owing to the increase of halogenation in the molecules. Therefore, modern pyrethroids are more persistent products, with greater power and longer residual half life. But these features also enhance the possibility of harmful effects occurring in aquatic systems [1–3].

There is little information available about the effects of pyrethroid on microorganism despite their ecological importance. Ninety six hours Cyfluthrin IC_{50} values for green algal species were greater than 10 mg/l [4] but in some cases, the degradation products of pyrethroids have been documented to be more toxic to bacteria, fungi and algae. The Permethrin EC_{50} value for algal growth in Cyanophyta *Anabaena* and some species of Chlorophyta as *Chlorella pyrenoidosa* was greater than 10 mg/l, while the EC_{50s} of the degradation products were between 1.4 and 8 mg/l. Similar results were found regarding effects on photosynthesis, with EC_{50} values greater than 100 mg/l for Permethrin and between 30 and 70 mg/l for the degradation products [5]. Studies on the effects of Cypermethrin and Fenvalerate on algal growth on several species of Cyanophyta and Chlorophyta indicated EC_{50} between 5 and 10 mg/l for both pyrethroids [6].

Most studies on algae used to assess the toxicity of these insecticides have been performing with technical grades of pyrethroids. Almost all studies conclude that pyrethroid insecticides would not cause harmful effects or that they would not be toxic to algae species present in freshwater environments. However, commercial formulations of these insecticides used in agricultural practices, contains constituents that provides a greater incorporation of the insecticide in target organisms. These constituents would be extremely harmful to natural algae populations, affecting algal physiology and growth compromising their development and presence in aquatic systems after exposure to pyrethroids commercial formulations.

In Argentina, 215.000 L of commercial formulation of Cyfluthrin were used in the last year, as it is applied over sucking and chewing insects that attacks a glyphosate tolerant transgenic variety of soybean which crops have reached to an extension of fourteen million hectares in the last year. Cyfluthrin represents 7% of the total amount of pyrethroid insecticides applied all over the country,

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being the fourth in importance (Argentine Crop Protection Association [7]). The application rates are between 150 and 400 cm^3 of commercial formulations per hectare, depending on the type of target organism. The treatment is conducted by direct application over the surface of leaves in spray using terrestrial or aerial equipment. Cyfluthrin is a contact insecticide that acts on insects that attack others crops such as cereals, corn, cotton, potatoes, rice, tobacco, sweet potatoes, wheat, sunflower, fruits, pea, cauliflower and apple. Cyfluthrin has a low solubility in water, in order of 2 μg/l. Its half-life is estimated at 30 days [8,9].

Given the important role of microalgae in aquatic systems and its essential function in nutrient cycles, the effects of one of the most widely used pyrethroid in Argentina, Cyfluthrin, were studied on green algae photosynthesis, growth and enzyme activity of catalase, as an indicator of damage to the antioxidant defense system. The insecticide was evaluated as a commercial formulation form. The hypothesis and aims of the study were as follows,

Hypothesis 1: Cyfluthrin commercial formulation is toxic to green algae species studied.

Hypothesis 2: Catalase enzyme is an earlier biomarker of adverse actions as its activity is modified at lower concentrations than those that affects growth and photosynthesis.

The aim of the study was to determine the effects of a commonly used Cyfluthrin commercial formulation in soybean crops on growth, physiological and biochemical parameter of green algae. Growth was chosen as a response that integrates several processes, photosynthesis as a specific physiological parameter and catalase enzyme activity as a first exploratory analysis of biochemical indicator of oxidative stress. Determine which of these different approaches would detect signs of toxicity so far in advance and have, in this way a predictive tool. Another objective of this study was to compare results from the traditional methodology of short-term chronic toxicity test of 96 h of exposition and a methodology based on the analysis of photosynthetic rate.

2. Materials and methods

2.1. Plant material

The organisms used were the Chlorophycean *Scenedesmus quadricauda* (native strain isolated from an upstream non polluted site of Luján river, Province of Buenos Aires. Identification was done according to Phycology Laboratory, Faculty of Natural Sciences and Museum, National University of La Plata), *Scenedesmus acutus* (SAG 273–3a), *Chlorella vulgaris* (Companhia de Saneamento Ambiental Estado de São Paulo, Brazil CETESB) and *Pseudokirchneriella subcapitata* (Korschikov) Hindák 1990 (CCAP 278/4), (formerly, *Selenastrum carpicornutum*). Algae was maintained in batch cultures containing 200 ml of mineral growth medium (pH 7,5) US EPA [10,11]. Algal culture of all species was grown under continuous illumination, (86 $\mu E/m^2/s$) provided by white fluorescent lamps (General Electric F15 W/54) at 24 ± 1 °C on an orbital shaker at 100 rotations per minute (rpm). Cultures were maintained in exponential growth by subculture every week.

Regular test with $K_2Cr_2O_4$ as reference toxicant were done as validity criteria in relation to the sensibility of the clones used.

2.2. Chemical substances

We used commercially formulated 5% cyfluthrin [Ciano-[4-fluoro-3-(fenoxi) fenil] metil]-3-(2,2dicloroetenil)-2,2-dimetilciclopropanocarboxilato) (Bayer Inc., Argentina). Solutions containing commercial formulation of Cyfluthrin were analyzed according to Di Marzio et al. [12]. In brief, insecticide was extracted twice with methanol (1:4) in an ultrasonic bath (Testlab1) for 60 min and then passed through C18 columns (Supelco). The pesticide analysis was performed after elution of the C18 columns with 2 ml hexane followed by 2 ml dichloromethane. The extracts were injected into a GC with mass spectrometer QP 5050A and Mass Spectrometry Workstation Class 5000 (Shimadzu GC/MS). Quantification was done using an external standard method purchased from Supelco. The limit of quantification was 10 μg/l. All reported Cyfluthrin concentrations are expressed as mg Cyfluthrin/l. All other chemicals included those used to measure enzyme activity were of analytical quality and purchased from commercial suppliers.

2.3. Algal growth bioassays

The toxicity of commercial formulation of Cyfluthrin was evaluated by algal growth inhibition test of 96 h using different species mentioned above. Algal culture medium was prepared by adding macro and micronutrient solutions to Milli-Q water. The pH of medium was adjusted to 7.5 (±1) using 0.1 N NaOH or HCl solutions as described in US EPA [11]. The resulting culture water had an average hardness of 200 mg/l (as $CaCO_3$) and an average alkalinity of 50 mg/l (as $CaCO_3$). The medium was filtered through a 0.45 μm pore diameter membrane in a vacuum. The medium was sterilised by autoclaving (120°, 15 min). The protocol for growth inhibition bioassays was based on standard procedures outlines in US EPA [11]. Experiments were conducted in 250 ml Erlenmeyer flasks containing 100 ml of sterilized culture media per replicate with four replicates per concentration per experiment. Nutrient medium was supplied with different pesticide concentrations prepared from commercial formulation. Test concentrations used in this study were selected based on previous range finding test. The appropriate volume of product was added to each flask from a single stock solution.

Each experiment was initiated using algae from cultures that were in an exponential growth phase and approximately 5–7 days old. Test flasks were inoculated with algal samples that had been concentrated to a density of 10000 cells/ml. The same initial density was used for all species. Commercial formulation assays were conducted under controlled conditions in a "Forma" incubation orbital shaker at 24 ± 1 °C, 86 $\mu E/m^2/s$ of continuous "cool-white" fluorescent lighting operating at 100 rotations per minute (rpm) [13]. Flask position on the shaker was randomized daily by treatment.

At the end of the exposure period, algal biomass were measured and dose response curves for each green algal species were obtained from experiments conducted as described above. Algal growth was the endpoint used based on total chlorophyll content measured during the incubation period. Samples were removed from the algal cultures and chlorophyll fluorescence were measured on a Turner TD 700 fluorometer (USA) using an excitation wavelength of 420 nm and measuring the emission at 680 nm.

The growth rate, μ (day^{-1}) was calculated as follows:

$$\mu = \frac{\ln(N_t - N_0)}{(t_t - t_0)}$$

where N_t the final biomass, N_0 the initial biomass, and $t_t - t_0$ the time (days) after the initiation of the test [14]

At 96 h of exposure, the percentage of growth inhibition with respect to the control culture was calculated as:

$$\%\ \mathrm{I} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{C}} \times 100$$

where C is the growth rate in the control and T is the growth rate in the presence of Cyfluthrin. Percentage of growth inhibition versus concentration values were plotted for each species.

The procedure of Payne and Hall [15] was used to determine algistatic (inhibition of growth) and algicidal (cell death) effects [10]. After the contact period of 96 h, only cultures with an

apparent inhibition of growth were centrifuged (10 min, 2000g) and the algal pellet was suspended in fresh nutrient medium and centrifuged again. The cells were counted and inoculated into sterile nutrient medium free of toxicant. Initial inoculum density was about 5×10^4 cells/ml. Recovery was evaluated during a period of ten days under the same incubation conditions of the contact period, but in the absence of toxicant. The enumeration of cells was performed on days 3, 5, 7, and 10 [15,16,10,13].

2.4. Enzyme assays and enzyme extraction

2.4.1. Enzyme preparation

The catalase (CAT) activity was studied in cultured algae exposed to Cyfluthrin commercial formulation. The algal suspensions of four algal species were incubated for 48 h in 100 ml of medium supplied with different concentrations of the pesticide prepared from the commercial formulation. The incubation conditions were those described above. After the exposure time, the cultures were collected by filtration through a 0.45 μm filter pore diameter. The filters were grounded 2 min in 1 g of quartz sand with the addition of 4 ml of 25 mM sodium phosphate buffer (pH 7) at 4 °C. The enzyme extraction was performed by sonication in cold buffer using three cycles of two minutes each at 70 W [17,18]. At the end of this procedure, the enzyme extract was separated from debris by filtration (Whatman GF/C).

2.4.2. Protein determination

Protein concentration was evaluated by the method of Lowry et al. [19] using bovine serum albumin as a standard.

2.4.3. Enzyme determination

Catalase (CAT) (1.11.1.6) activity was determined spectrophotometrically by measuring the consumption of H_2O_2 at 240 nm (ε: 0036 mM^{-1} $cm^{-}1$) in a reaction medium containing 50 mM sodium phosphate buffer (pH 7.3), 100 mM of H_2O_2 and enzymatic extract [20,21].

2.5. Oxygen production rate

The algal cultures used in the photosynthetic oxygen production tests, were maintained in a climatized chamber at 20 ± 0.5 °C, exposed to a light intensity of 50 Watt/m^2 with a photoperiod of 14 h light/10 h darkness placed on a continuous orbital shaking at 100 rpm. EPA [11] nutrient medium was used for the algal cultures of four species.

The total rate of photosynthetic oxygen production was performed using a polarographic Clark type oxygen sensor (Hansatech, Kings Lynn, UK) [22]. A methodology was developed to implement this instrument in micro algae ecotoxicological studies identifying the most appropriate conditions of light, algal density and age of the cultures to be used in the oxygen production tests. A detailed description of the method and the procedures used in the establishing of the experimental conditions can be found in Sáenz [13].

The electrode was equipped with a cylindrical chamber measuring 15 mm inner diameter and 2.5 ml capacity with a stirrer bar at its base, provided of an outer jacket for thermostat-controlled water. The algal suspension derived from the culture vessels was immediately introduced into the chamber. After a few seconds, necessary for the adaptation of microalgae to the measuring chamber, the total rate of oxygen production was recorded. The cell suspension inside the chamber was continuously stirred while measurements were taken. Temperature was of 20 ± 0.5 and irradiance of 100 Watt/m^2 inside the chamber provided by an optic fibre light through a jacket on one side of it. A quantum-radiophotometer Licor model Li 185 was used to measure irradiance. The instrument was connected to a computer through an interface that allowed the calculations of the rate of photosynthetic oxygen production in a continuous manner, so the kinetic of the process could be determined.

Light saturation curves were constructed for each species. The kinetics of light saturation were fitted to the Michaelis–Menten model:

$$\text{Photosynthetic rate} = \frac{\text{Fmax} * \text{Light intensity}}{\text{I m} + \text{Light intensity}}$$

where:Fmax: maximum photosynthetic rate

I m: light intensity at which the half of the maximum photosynthesis rate is recorded.

The average inhibition was calculated by comparing the oxygen production rate of the cultures exposed to the different concentrations of Cyfluthrin formulation with the oxygen production rate of control cultures (not exposed).

The application of this methodology in assessing the effects of some herbicides on green algae photosynthesis can be found in Mingazzini et al. [23].

2.6. Data analysis

Threshold concentrations including the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) were estimated using traditional hypothesis testing techniques. Normality of data was first assessed using the Shapiro–Wilks test and homogeneity of variance was assessed with the Bartlett's test. The NOEC and LOEC were determined using Dunnett's test, a parametric test for comparing individual treatment concentrations against controls [24,25]. A linear interpolation method was used to estimate inhibition concentrations (IC) for algal growth and oxygen production rate, using ICPIN software (Version 2.0 (US EPA Duluth MN USA).

One-way ANOVA was used to assess the differences in algal growth, photosynthesis rate and enzymatic activity in conjunction with Tuckey's multiple range tests and Dunnett's test ($p < 0.05$). All statistical analysis was performed using Statistica v. 8 (StatSoft).

Recovery data were processed according to Payne and Hall [15], US EPA [10] and Ricker [26].

3. Results

3.1. Effects on algal growth

Concentrations of 0.6 mg Cyf/l exerted a non-significant inhibition of *S. quadricauda* growth with respect to the controls at 96 h of exposition. At this time the growth of the algae cultures was reduced significantly at insecticide concentration above 1 mg Cyf/l. When algae cultures were grown in the absence of insecticide during the recovery period, all cultures resumed the exponential growth with the exception of those that had been exposed to the highest concentration (4.6 mg Cyf/l). This concentration exerted algicidal effects since algae cultures did not grow exponentially. The estimated algistatic concentration of commercial formulation was 3.73 mg Cyf/l (Fig. 1).

Growth cultures of *S. acutus* was not significantly affected at 0.03 mg Cyf/l. The algal growth was significantly reduced at insecticide concentrations ranging from 0.1 to 14.4 mg Cyf/l (ANOVA – Dunnett test–Tuckey test $p < 0.05$). During the recovery period algal cultures preexposed at concentrations between 0.03 y 1.3 mg Cyf/l developed an exponential growth as the controls. Cultures exposed to 4.4 mg Cyf/l also exhibited an exponential growth but these had a lower growth rate thereby producing a different growth pattern. The growth of the algal cultures preexposed to

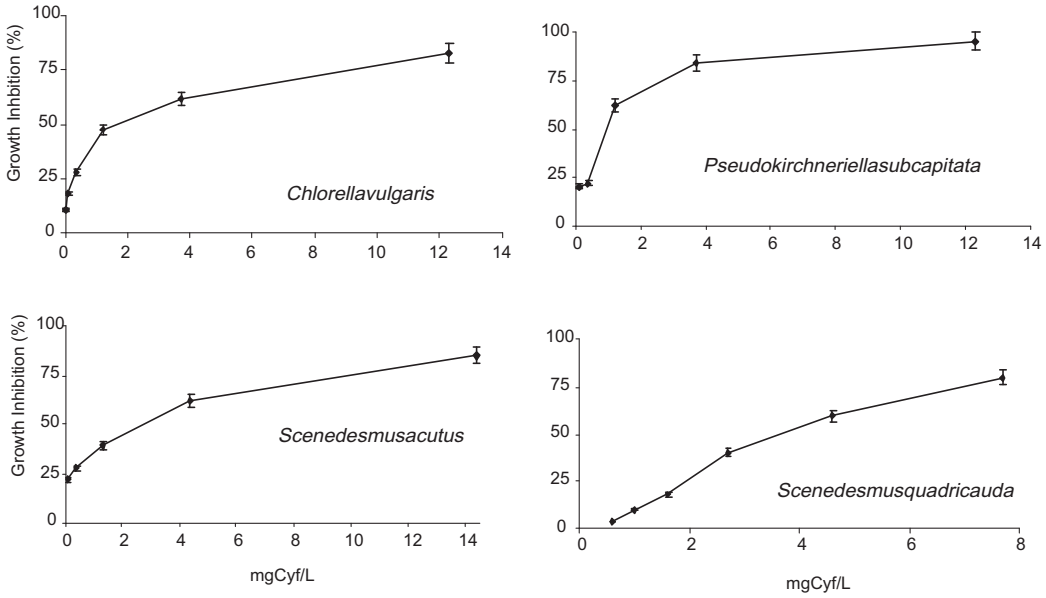

Fig. 1. Inhibition growth respect to the control of algal cultures of different species used, after 96 h of exposition to different concentrations of Cyfluthrin commercial formulation.

14.4 mg Cyf/l showed an extended lag phase during the first three days; after that an incipient increase of cell density was observed between days five and seven, but a notable reduction of growth was achieved at the end of the recovery period. These results indicates that concentrations of Cyfluthrin commercial formulation between 0.03 y 4.4 mg Cyf/l produced algistatic effects since the inhibition of growth during the contact period were reversible following exposure to the insecticide -free nutrient medium. On the other hand, an algicidal response was observed in those cultures exposed to 14.4 mg Cyf/l. At this concentration Cyfluthrin commercial formulation exerted irreversible and permanent harmful to algal cells. The estimated algistatic concentration was 1.89 mg Cyf/l (Fig. 1).

In the case of *C. vulgaris* all the assayed concentration equals and higher for 0.03 mg Cyf/l significantly inhibited growth relative to the control cultures at 96 h of treatment (ANOVA–Tuckey test $p < 0.05$). During the recovery period growth of algal cultures exposed to concentrations between 0.03 and 3.7 mg Cyf/l were not significantly different from the control cultures (ANOVA–Dunnett test $p > 0.05$). Concentrations of 12.3 mg Cyf/l significant affected the growth recovery of algal cultures since these did not show exponential growth. These results indicate that Cyfluthrin commercial formulation exerted algicidal effects at concentrations of 12.3 mg Cyf/l, confirming the algicidal response observed during the contact period. The estimated algistatic concentration of Cyfluthrin commercial formulation to *C. vulgaris* was 3.48 mg Cyf/l (Fig. 1).

Cyfluthrin commercial formulation did not affect significantly algal growth of *P. subcapitata* compared to the control at a concentration between 0.03 and 0.37 mg Cyf/l after 96 h of exposure. Concentrations ranging from 1.2 to 12.3 mg Cyf/l produced a significant inhibition of growth with respect to the control. During the recovery experiences, similar results to *C. vulgaris* were found. Cultures of *P. subcapitata* preexposed to the same range of concentrations (0.03 and 3.7 mg Cyf/l) also growth as the control cultures. Cultures preexposed to 12.3 mg Cyf/l did not recover from Cyfluthrin exposure. Commercial formulation of Cyfluthrin was algicidal at concentrations of 12.3 mg Cyf/l. The concentration that caused algistatic effects on *P. subcapitata* was 2.55 mg Cyf/l (Fig. 1).

Toxicity endpoints derived from growth parameters are shown in Table 1. Statistical analysis of 96 h IC_{50} values indicates that *P. subcapitata* was the most sensitive specie. *S. quadricauda* was the least sensitive featuring the highest value. Taking into account chronic toxicity endpoints there were no differences between NOEC and LOEC estimated for *S. acutus*, *C. vulgaris* and *P. subcapitata* while *S. quadricauda* showed the highest values (Table 1). IC_{25} values showed similar results as there was no significant difference between *S. acutus*, *C. vulgaris* and *P. subcapitata* indicating a similar sensitivity to Cyfluthrin commercial formulation. The IC_{25} value of *S. quadricauda* was the highest, in correspondence with the others endpoint estimated.

Table 1
Toxicity of Cyfluthrin commercial formulation to four algal species after a 96 h treatment based on growth measurements (mg/l).

Species	IC_{25} (95% CI)	IC_{50} (95%CI)	NOEC	LOEC
S. quadricauda	2.02 (1.60–2.37)	4.85 (4.47–5.13)	1.6	2.7
S. acutus	0.32 (0.23–0.40)	1.25 (1.12–1.67)	0.03	0.1
C. vulgaris	0.33 (0.30–0.37)	2.06 (1.77–2.29)	0.03	0.1
P. subcapitata	0.39 (0.23–0.51)	0.92 (0.80–1.02)	0.03	0.1

3.2. Effects on photosynthetic oxygen evolution

The photosynthetic oxygen rate was stimulated in algal cultures of *S. quadricauda* exposed to 0.01 and 1 mg Cyf/l for the first 40 min. With longer exposure times, a not significant inhibition of the photosynthetic rate was observed. Concentrations of 10 mg Cyf/l caused a significant inhibition of the photosynthetic rate of algal cultures since the beginning of the experience. In regards to the algal cultures exposed to the highest concentration assayed (100 mg Cyf/l) there was no photosynthetic oxygen production by the algal cultures at any time during the assay (Fig. 2A).

After 40 min of exposition of *P. subcapitata* cultures to 0.01 and 0.1 mg Cyf/l the photosynthetic rate was not significantly affected. In contrast, cultures exposed to 1 and 100 mg Cyf/l showed a significant inhibition of the photosynthesis rate. When the exposure time was increased to 50 min, concentrations higher than 0.1 mg Cyf/l significantly inhibited photosynthetic rates compared to the controls, while those exposed to 0.01 mg Cyf/l were not affected.

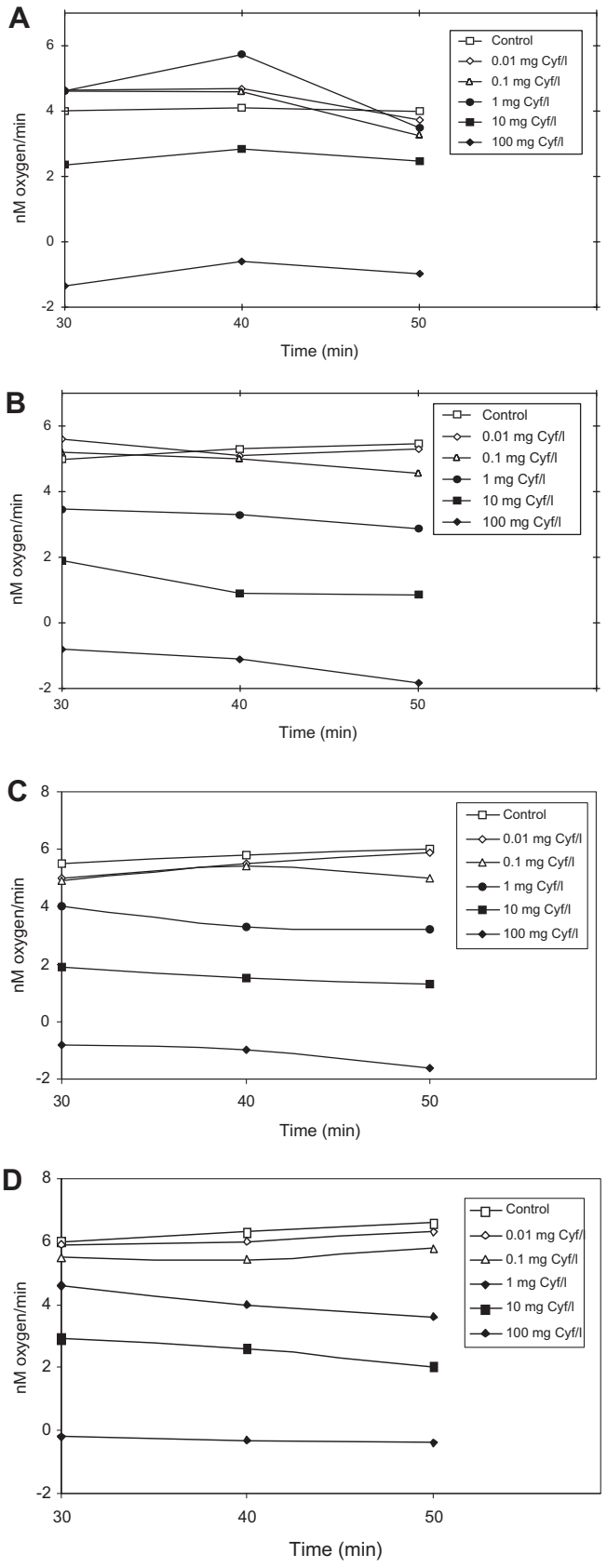

Fig. 2. Oxygen evolution of cultures of *S. quadricauda* (A), *P. subcapitata* (B) *S. acutus* (C) and *C. vulgaris* (D) exposed to different concentrations of Cyfluthrin commercial formulation during exposition time of 50 min.

Table 2
IC 50 values and confidence limits of commercial formulation (mg Cyf/l) to algae species after 50 min of exposition based on photosynthesis rate measurements.

Species	IC_{50} 50 min.	CI
S. quadricauda	8.9	6.54–12.3
S. acutus	2.1	1.2–2.9
C. vulgaris	3.4	3–3.8
P. subcapitata	1.7	0.9–2.8

Algal cultures exposed to 100 mg Cyf/l did not photosynthesize at any time during the assay (Fig. 2B).

Cultures of *S. acutus* were affected in the similar manner as described above. At 50 min of exposition concentrations from 1 to 100 mg Cyf/l significantly affected photosynthetic oxygen rate. Those cultures exposed from 0.01 to 0.1 mg Cyf/l did not showed a significant decrease in this parameter respect to controls (Fig. 2C).

The photosynthetic oxygen rate of *C. vulgaris* cultures exposed to Cyfluthrin commercial formulation was significantly inhibited since 40 min till the end of the exposition time, at concentration above 1 mg Cyf/l. This parameter was not affected in algal cultures exposed to 0.01 and 0.1 mg Cyf/l respect to control values (Fig. 2D).

The assessment of the effects of Cyfluthrin commercial formulation upon the photosynthesis process on algal species showed that IC_{50} after 50 min of exposition of *P. subcapitata* and *S. acutus* were significantly lower than the respective values for *S. quadricauda* and *C. vulgaris* (Table 2).

3.3. Effects on catalase activity

Enzymatic activities of four species exposed to Cyfluthrin commercial formulation are shown in Fig. 3. After 48 h of exposition CAT activity of those cultures of *S. quadricauda* exposed to 0.01 and 0.6 mg Cyf/l were significantly stimulated by 23% and 28% respectively relative to controls ($p < 0.05$). CAT activity was significantly lower in those cultures exposed to 1 mg Cyf/l compared to control, with an inhibition of 18%. As is shown in Fig. 3B cultures of *P. subcapitata* treated with the Cyfluthrine commercial product at 0.01 mg Cyf/l showed a significant increase in CAT activity ($p < 0.05$) of 33% compared with controls. On the contrary, at higher concentrations of 0.30 and 1 mg Cyf/l, enzyme activity was significantly lower than that of the control, resulting in an inhibition effect of 22% and 29%, respectively. CAT activities of cultures of *S. acutus* and *C. vulgaris* treated with the highest concentrations, also decreased respect to control. In the case of *S. acutus* the inhibition was of 25% and 32% respectively. For *C. vulgaris* these values were 22% and 33%. At 0.01 mg Cyf/l a significant increase in CAT ($p < 0.05$) activity was recorded for both species, being of 18% and 31%, respectively (Fig. 3C, Fig. 3D).

Action on CAT activity was significantly different from control at a concentration of 0.01 mg Cyf/l for four algal species after 48 h of exposure. This concentration is lower than LOEC values derived from algal growth inhibition test of 96 h of exposure (Table1) and also lower than IC_{50s} endpoints derived from photosynthesis determinations after 50 min of exposure (Table 2); therefore effects would be detected through alterations in enzyme activity prior and at much lower exposure concentrations to those affecting algal growth and photosynthesis, resulting in a more sensitive parameter.

In this study, *P. subcapitata* was the most sensitive species to the action of the Cyfluthrin commercial formulation considering both effects on growth and on photosynthetic process. CAT activity as an indicator of oxidative stress damage was severely inhibited at

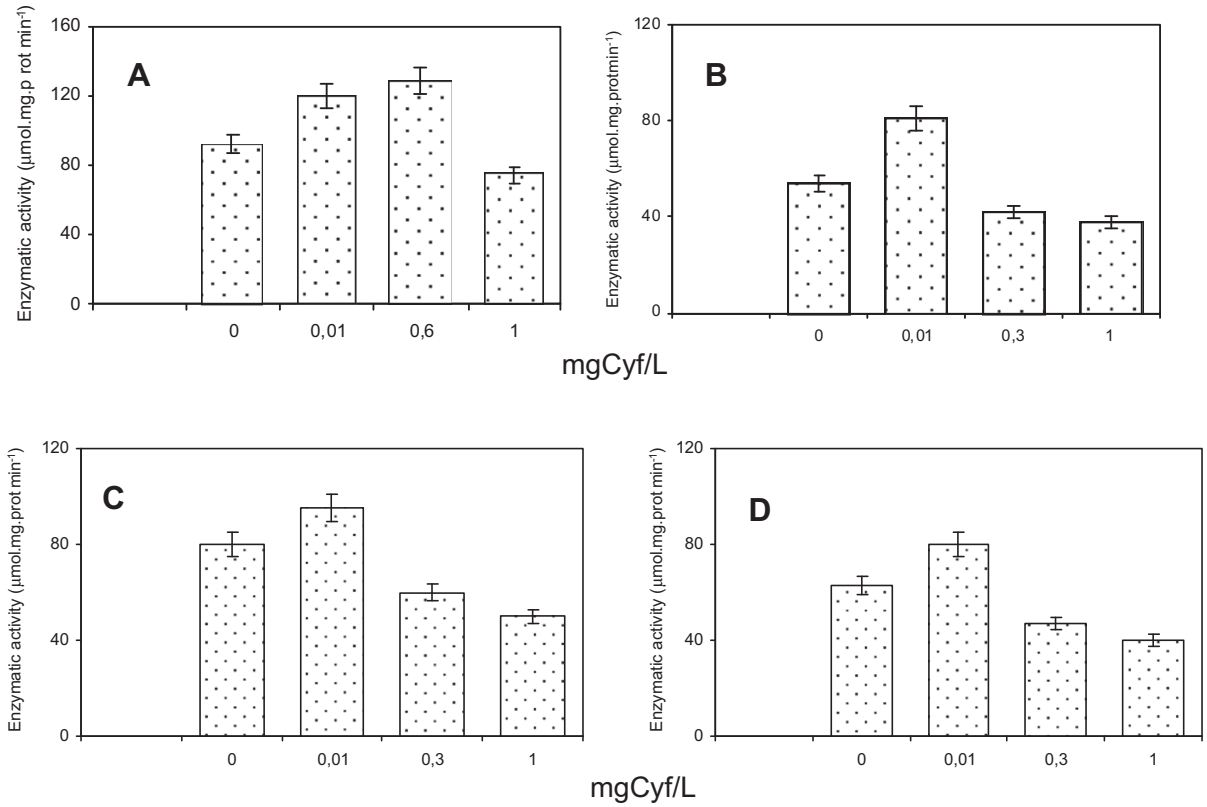

Fig. 3. Effect of Cyfluthrin commercial formulation on catalase activity of *S. quadricauda* (A), *P. subcapitata* (B) *S. acutus* (C) and *C. vulgaris* (D) after 48 h of exposure (*$p < 0{,}05$).

a concentration lower than that affecting *S. quadricauda.* In this case, sensitivity was similar to the others two algal species considered, *S. acutus* and *C. vulgaris*.

4. Discussion

The ecotoxicological assessment of Cyfluthrin commercial formulation on algal species used showed that this insecticide caused serious effects on algal growth. Studies carried out to assess algal recovery after exposure to the insecticide indicated that natural algal populations would be severely affected after exposure to the pyrethroid insecticide Cyfluthrin as the commercial formula. This product exerted algistatic and algicidal effect upon*S. quadricauda*,*S. acutus*,*C. vulgaris* and*P. subcapitata*. This is an important matter to consider since after such environmental concentrations natural algae population would have permanent, irreversible damage and would have no resilience. Cyfluthrin commercial formulation affected algal photosynthesis process. This means that after a short exposure of at least 40 min algal photosynthetic process also would be affected. The effects of stimulation of photosynthesis registered in the case of *S. quadricauda* have also been found in studies with the herbicide Glyphosate [27]. Paraquat caused stimulatory effects on green algal growth [28]. These phenomena have been pointed out by others authors [29]. Low concentrations of pesticides would cause cellular stress and imbalance at the cellular level producing a stimulation of different processes as a first sign of disturbance of biological processes. At higher concentrations the damage would be more drastic causing an inhibition of the photosynthesis rate. This phenomenon has been described over a century ago as the Arndt-Schulz law, being introduced the term "hormesis" in 1943 by Southam and Erlich. The hormetic dose response may be best assessed within the context of a dose-time response since the low dose stimulation response often represents a modest overcompensation response following an initial disruption in homeostasis (toxicity). As time progresses a compensatory response occurs at low doses eventually leading to the low dose stimulation characteristic of hormesis. Such compensatory responses are not usually achieved at the higher doses, leading to the hormetic biphasic dose response. The nature of the overcompensation response appears to result from biological compensatory processes that allocate resources slightly in excess of those insuring a return to homeostasis. This 'extra' allocation of resources leads to the hormetic stimulation. This accounts for why hormesis is best observed within the context of a dose-time effect evaluation [30]. A recent study of terrestrial and aquatic vascular plants and algae exposed to low doses of ten herbicides has shown that there are some herbicides that cause mild hormesis phenomena, while others cause more pronounced responses [31]. The cyfluthrine commercial product would cause a low hormetic response.

Regarding the difference in sensitivity between the green algae species used in this study, *P. subcapitata* was the most sensitive specie to the action of the Cyfluthrin commercial formulation. This fact was considering both the effects on growth population and the effects on photosynthetic rate. The greater sensitivity of this specie would be associated to a faster penetration of the formulation in virtue of a one layer, therefore a simpler constitution of its cell wall compared to the complex one of the species belonging to genus *Scenedesmus* [32].

An important conclusion also to point out is that similar sensitivity endpoints were found between the four species studied as a consequence of applying the traditional methodology of short-term chronic toxicity test of 96 h of exposition and the methodology developed using the Clark type photosynthetic oxygen evolution method. This methodology allowed detect in a faster manner effects at concentrations ranging in the same order of magnitude that those detected by the algal growth inhibition test of 96 h of exposition (IC_{50} 50 min for photosynthetic rate: 1.7–8.9 mg Cyf/l; IC_{50} – 96 h for algal growth = 0.92–4.85 mg Cyf/l). These findings were also

reported by Mingazzini et al. [23] when this methodology was applied to study the effects of two herbicides Atrazine and DCMU on the same algae species. The advantage of the developed methodology consists of rapid achieving of toxicological information which is relevant in monitoring programs and pesticide risk assessment allowing to have a first preliminary information about the damage towards an autotrophic planktonic community of freshwater environments. Indeed this is a really fundamental fact in virtue of the important amounts of pesticides used in relation to the fourteen million hectares cultivated with glyphosate tolerant transgenic variety of soybean all over the country.

Studies carried out with technical grades of pyrethroids conclude that they are of low toxicity compared with others insecticides [33]. Respect to antioxidative enzymes Xiong et al. [33] have showed that concentrations as high as 100 mg/l of Cypermetrin technical grade stimulated SOD activity of *S. obliquus* as a way of eliminate active oxygen in algal cells when the concentrations of this xenobiotic increases. This threshold is much higher than those reported here from pyrethroid commercial formulations. Previous studies (in press) about ecotoxicological assessment of Cypermethrin as commercial formulation, towards the same algae species used in this study, have shown growth inhibition, algistatic and algicidal effects and an increase of oxidative stress enzymes, catalase and glutathione reductase. Toxicity endpoints were very similar to the ones reported here for Cyfluthrin commercial formulation. These similar results would indicate a similar toxicity mechanism towards green algae of these insecticides. As was pointed out, commercial formulation of insecticides pyrethroids contains xylene in order to increase the effectiveness to the target organisms. Toxicity assessment of this solvent to the algae species used in this study shown that the 96 h IC_{50s} were between 0.10 and 0.38 mg/l [13]. So this fact would explain part of the higher toxicity of both pyrethroid commercial formulations, Cypermethrin and Cyfluthrin. One important matter to considering regarding the environmental risk of these insecticides is that, in spite of the fact that technical grade of pyrethroids does not provoke toxic effects to algae, [6–13] commercial formulations indeed would affect algal growth, growth kinetics, important vital processes such as photosynthesis reactions and ROS generation due to the addition of organic solvent in the formulations. In this sense, the initiation of synthesis of antioxidant enzymes and their activation would be stimulated as a response to cellular antioxidative mechanism. Particularly catalase enzyme is part of the plant defense when oxidative stress leads to lipid peroxidation, protecting cellular membranes against ROS. It is located mainly in cytosol, in which the importance in hydrogen peroxide metabolism and scavenges is determinant in the hability of cells to cope with ROS. Stimulation and activity of this enzyme was often reported in plants exposed to oxidative stress generating compounds [34]. So, from our results we can assume that low concentrations of pyrethroid formulations strongly stimulated CAT enzyme of green algae studied. This phenomena is probably attributable to strengthening of antioxidative defenses. The decrease in CAT activity observed for the four algal species at higher concentrations can be explained by alteration of protein integrity as a consequence of peroxidative destruction as well as disintegration of cellular membranes systems by lipid peroxidation caused by the presence of solvent in the formulation. Due to an overproduction of ROS the activity of the enzyme was not enough to cope with the excess of hydrogen peroxide generated by formulations. These changes took place at the molecular level in the cells, so this is the reason why they happened much earlier than growth or reproduction.

Commercial formulations are those that are finally used in aquatic system and are in close contact to the aquatic organisms. In this context is worthwhile to point out that activation of antioxidant enzymes could be used as a sensitive biomarker for early warning of the action of these commercial products. Biomarkers are important supplementary approach, they provide a measure of the operation of a toxic mechanism and can give evidence that a chemical existing in the environment is actually operating in the living organism and be responsible of population decline. A biomarker can give an initial signal that after leads to physiological and biochemical disturbances that become manifest at the whole organism level. As a consequence, this can lead to adverse effects at the population level [3]. Such an approach can give powerful evidence of causality; it may also have an important role in the risk assessment of environmental chemicals. Wendt-Rasch et al. [35] and Friberg-Jensen et al. [36] have reported important harmful effects on crustacean zooplankton and alterations in species composition of autotrophic communities such as periphyton and phytoplankton in freshwater community under field conditions after applications of pyrethroid insecticides commercial formulation. So these products would affect the entire aquatic community. Cyfluthrin concentrations of 5–8 μg/l have been found in surface waters as a result of agricultural practice [37]. These concentrations could be higher depending on rainfall intensity and the amount of water leaving the fields via surface runoff. Heavy rain occurs during pampasic summer which is the season where transgenic soybean is grown. That means that the extremely large volume of water produced in a short time being runoff the main route of entry. Entrance to aquatic systems by spray drift is also important, as most of the time aerial applications are used due to the large extensions to be treated with insecticides. It would be important the definition and implementation of risk mitigation strategies and improved measures of mitigation capabilities as it might make possible to adapt the agricultural practice and pesticide application on a local level, e.g. reducing or differentiating distance between sprayed fields and surface waters for specific compounds. In this scenario we can conclude that CAT activity would be a sensitive biochemical biomarker, offering fast and reliable indication of aquatic pollutants toxicity impact, as at 10 μg/l significant effects was observed. It could be considered an important tool in detecting harmful effects prior to worst effects on growth and photosynthesis observed at higher exposure concentrations.

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References

[1] J.P. Leahey, The pyrethroid insecticides, Taylor & Francis, NY, 1985.
[2] S.P. Bradbury, J.R. Coats, Comparative toxicology of the pyrethroid insecticides, Rev. Environ. Contam. Toxicol. 108 (1989) 134–177.
[3] C.H. Walker, S.P. Hopkin, R.M. Sibly, D.B. Peakall, Principles of Ecotoxicology, Taylor & Francis, 1996.
[4] FAO specifications and evaluations for agricultural pesticides, Cyfluthrin, 2003.
[5] G.W. Stratton, C.T. Corke, Toxicity of the insecticide permethrin and some degradation products towards algae and cyanobacteria, Environ. Pollut. 29 (1982) 71–80.
[6] A.E. Pipe, Pesticides effects on soil algae and cyanobacteria, Rev. Environ. Contam. Toxicol. 127 (1992) 95–170.
[7] CASAFE Cámara de Sanidad Agropecuaria y Fertilizantes. Buenos Aires, Argentina. http://www.casafe.org/.2009 Access May 2011.
[8] Royal Society of Chemistry, The agrochemical handbook of the Royal Society of Chemistry. Nottingham, England 1987.
[9] A.G. Hornsby, R. Don Wauchope, A.E. Herner, Pesticide properties in the environment. Springer-Verlag, New York, Inc., 1996.
[10] US EPA (United State Environmental protection Agency), Ecological Test Guidelines. OPPTS 850.5400. Algal Toxicity Tiers I and II. Prevention Pesticides and Toxic Substances, EPA 712-C-96-164, 1996.
[11] US EPA (United States Enviromental Protection Agency), Selenatrum capricornutum growth test. In: Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms Fourth Edition October, EPA-821-R-02-013, 2002.
[12] W.D. Di Marzio, M.E. Sáenz, J.L. Alberdi, N. Fortunato, V. Capello, C. Montivero, G. Ambrini, Environmental impact of insecticides applied on biotech soybean

crops in relation to the distance from aquatic ecosystems, Envir. Toxicol. Chem. 29 (2010) 1907–1917.
[13] M.E. Sáenz, Estudios de los efectos de contaminantes sobre poblaciones algales de agua dulce, Ph.D.Thesis, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata. 2000.
[14] Organization for Economic Cooperation and Development, Alga growth inhibition test. Guideline 201, Paris, France, 2002.
[15] A.G. Payne, R.H. Hall, A method for measuring algal toxicity and its application to the safety assessment of new chemicals, in: L.L. Marking, R.A. Kimerle, (Eds.), Aquatic Toxicology, American Society for Testing and Materials ASTM STP 667, 1979, pp. 171–180.
[16] G.R. Rand, Fundamentals of aquatic toxicology: effects, environmental fate and risk assessment, Taylor & Francis, NY, 1995.
[17] E.A. Meijer, R.H. Wiiffels, Development of a fast, reproducible and effective method for the extraction and quantification of proteins of micro-algae, Biotechnol. Tech. 12 (1998) 353–358.
[18] L. Woods, M. Riccobono, N. Mehan, J. Hestekin, R. Beitle, Synergistic effect of abrasive and sonication for release of carbohydrate and protein from algae, Sep. Sci. Technol. 46 (2011) 601–604.
[19] O.H. Lowry, N.J. Rosembrough, A.L. Farr, A.J. Randal, Protein measure with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[20] R.F. Beers Jr, I.W. Sizer, A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase, J. Biol. Chem. 195 (1952) 133–140.
[21] F. Wu, G. Zhang, P. Dominy, Four barley genotypes respond differently to cadmium: lipid peroxidation and activities of antioxidant capacity, Envir. Exp. Bot. 50 (2003) 67–78.
[22] D. Walker, The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis. Research Institute for photosynthesis, University of Sheffield, UK, 1987.
[23] M. Mingazzini, M.E. Sáenz, F.G. Albergoni, M.T. Marré, Algal photosynthesis measurements in toxicity testing, Fres. Environ. Bull. 6 (1997) 308–313.
[24] T. Sparks, Statistic in Ecotoxicology. Ecological and Environmental Toxicology Series. John Wiley & Sons, New York, USA, 2000.
[25] J.H. Zar, Biostatistical Analysis, Pearson Boston, MA, USA. 2010.
[26] W.E. Ricker, Linear regressions in fishery research, J. Fish. Res. Board Canad. 30 (1973) 409–434.
[27] M.E. Sáenz, W.D. Di Marzio, Ecotoxicidad del herbicida glifosato sobre cuatro algas dulceacuicolas, Limnética 28 (2009) 1–10.
[28] M.E. Sáenz, J. Accorinti, M.D.C. Tortorelli, Toxicity of paraquat to a green alga *Scenedesmus acutus*, J. Environ. Science and Health – Part B. Pesticides, Food contaminants and agricultural waste, 28 (1993) 193–204.
[29] N. Nyholm, T. Källqvist, Methods for growth inhibition toxicity test with freswater algae, Environ. Toxicol. Chem. 8 (1989) 689–703.
[30] E.J. Calabrese, Paradigm lost, paradigm found: the re-emergence of hormesis as a fundamental dose response model in the toxicological sciences, Environ. Pollut. 138 (2005) 378–411.
[31] N. Cedergreen, J.C. Streibig, S.K. Mathiassen, S.O. Duke, The occurrence of hormesis in plants and algae, Dose-Response 5 (2007) 150–162.
[32] E. Hegewald, F. Hindak, E. Schnepf, Studies on the genus *Scenedesmus Meyen* (Chlorophycaea, Chlorococcales) from South India, with special reference to the cell wall ultraestructure, Nov. Hedwigia Beih. 99 (1990).
[33] L. Xiong, P. Xie, X Sheng, Z. Wu, L. Xie, Toxicity of cypermethrin on growth pigments and superoxide dismutase of *Scenedesmus obliquus*, Ecotox. Environ. Saf. 60 (2005) 188–192.
[34] M.A. Torres, M.P. Barros, S.G. Campos, E. Pinto, S. Rajamani, R. Sayre, P. Colepicolo, Biochemical biomarkers in algae and marine pollution: a review ecotox, Environ. Saf. 71 (2008) 1–15.
[35] L. Wendt-Rasch, U. Friberg-Jensen, P. Woin, K. Christoffersen, Effects of the pyrethroid insecticide, cypermethrin, on a freshwater community studied under field conditions. II. Direct and indirect effects on the species composition, Aquat. Toxicol. 63 (2003) 373–389.
[36] U. Friberg-Jensen, L. Wendt-Rasch, P. Woin, K. Christoffersen, Effects of the pyrethroid insecticide, cypermethrin, on a freshwater community studied under field conditions. I. Direct and indirect effects on abundance measures of organisms at different trophic levels, Aquat. Toxicol. 63 (2003) 357–371.
[37] A. Shultz, Field studies on exposure, effects, and risk mitigation of aquatic nonpoint-source insecticide pollution: a review, J. Environ. Qual. 33 (2004) 419–448.